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Sublethal Effects of Methylmercury on Flight Performance and Molt in European Starlings (*Sturnus vulgaris*)

Jenna Rae Carlson

College of William & Mary - Arts & Sciences

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Sublethal Effects of Methylmercury on Flight Performance and Molt in European
Starlings (*Sturnus vulgaris*)

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Bachelor of the Arts, New York University, 2009

A Thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

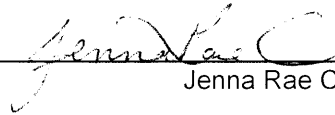
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APPROVAL PAGE

This Thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science



Jenna Rae Carlson

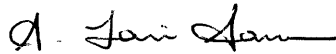
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COMPLIANCE PAGE

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ABSTRACT

The effects of methylmercury (MeHg) on the health of songbirds have not been adequately studied despite ample evidence that mercury bioaccumulates in terrestrial ecosystems. I performed the first analysis of flight performance and molt in captive MeHg-dosed European Starlings (*Sturnus vulgaris*). I dosed 60 European Starlings with methylmercury-cysteine (MeHgCys) through continuous dietary intake at 0.0 ppm, 0.75 ppm, or 1.5 ppm over the course of twelve months. To test flight performance, I measured: (1) Take-off angle and speed combined into a metric of energy gained during flight in response to a predator stimulus; and (2) velocity and angle while maneuvering around an obstacle in the flight path. I quantified molt using the Ginn and Melville method (1983). My results demonstrate that 40 weeks after treatment began, birds in the 0.75 ppm MeHgCys group exhibited a mean 17.2% decrease in energy during escape take-off relative to birds in the control group and birds treated with 1.5 ppm MeHgCys exhibited a 30.1% mean decrease relative to controls ($F_{2,40} = 3.80$, $P = 0.031$). Birds with higher blood mercury also molted more quickly than controls ($y = 0.025x + 0.29$, $F_{1,40} = 5.097$, $r^2 = 0.631$, $P = 0.030$). Mercury had no consistent effect on velocity or angle of turn while birds navigated through the maneuverability course ($F_{2,41} = 0.446$, $P = 0.644$; $F_{2,41} = 1.33$, $P = 0.277$, respectively). Overall, my results demonstrate that sub-lethal levels of mercury can cause abnormal molt patterns and reduced energy exertion during escape take-off flight. Inadequate flight performance may decrease fitness by reducing the ability to escape predators or by causing deficiencies in any number of vital behaviors in a bird's life that require efficient locomotion. This research is aimed at beginning to fill in the knowledge gap concerning risk thresholds for terrestrial songbirds, and indicates that 0.75 ppm and 1.5 ppm dietary mercury may negatively affect flight performance and molt in exposed songbirds.

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This MS is dedicated to animals used in scientific research, especially the
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INTRODUCTION

Section I: The Biology of Songbird Mercury Exposure

i. Behavior of mercury in the environment

Mercury (Hg) is an element that occurs naturally throughout the world largely in mercuric sulfide deposits (cinnabar). It is the only metal that is liquid at standard temperature and pressure and it is therefore used in a wide range of industrial applications. This industrial use has led to increased mercury deposition into air, soil, and water, with subsequent redistribution and mobilization through food chains and ecosystems (Boenig 2000).

Mercury is toxic in both inorganic and organic compounds but its organic forms are the most bioavailable (Boening 2000; Burcher et al. 2006). Inorganic mercury, often the form released by anthropogenic activity, is converted into organic forms by anaerobic microorganisms in the environment (Wolfe et al. 1998). Chloride and sulfide bind to ionic mercury (Hg (II)) and create a neutral species than can cross the cell membrane of bacteria, where a methyl group donor can methylate the mercury, creating methylmercury (MeHg). Anoxic environments with abundant sulfur, aluminum, and low pH catalyze this process. Combinations of these characteristics can often occur in aquatic environments and can lead to abundant conversion of inorganic mercury into bioavailable forms.

Methylation can also occur outside of bacteria through abiotic reactions. For example, industrial wastewater and sewage effluent can catalyze methylation of inorganic mercury (Ulrich et al. 2001). It is well known that MeHg bioaccumulates and biomagnifies as it

moves up the food chain in aquatic habitats (Wolfe et al. 1998; Boening 2000), but this process can also occur in terrestrial ecosystems (Evers et al. 2005; Rimmer et al. 2005; Driscoll et al. 2007). MeHg bioaccumulation has been shown to have deleterious effects on aquatic and terrestrial vertebrates, invertebrates, and plants alike (Boening 2000; Scheulhammer 2000; Seewagen 2010).

ii. Mercury as a global pollutant

Over the past two decades, anthropogenic mercury deposition into air, water, and soil has doubled or tripled global mercury fluxes (Driscoll et al. 2007). Some sources of Hg pollution include gold and silver mining, industrial effluent, agricultural drain water, decomposing organic matter behind dams, and burning of fossil fuels. Approximately 50% of atmospheric mercury is the result of human activity (Pacyna et al. 2006; Wilson et al. 2006). Combustion of fossil fuels, primarily coal, for energy generation is an especially significant source of pollution, producing one-third of the total atmospheric mercury (Pacyna et al. 2006; Wilson et al. 2006). Atmospheric mercury is mobilized through trade winds and jet streams and can accumulate thousands of miles from its source. For example, the Adirondack Mountain region in northeastern New York is considered a biological “hotspot” due to mercury accumulation stemming from coal plants in the Midwest (Adams et al. 2009; Selvendiran et al. 2009). Mercury has even been detected in Antarctica, where there are no proximate anthropogenic sources (Wilson et al. 2006). In the USA some progress has been made in reducing atmospheric mercury at the state level, for example Illinois plans to reduce emissions of Hg from coal by 90% by the year 2018 (Illinois Clean Air Interstate Rule). However, mercury persists

throughout ecosystems decades after deposition. In one well-documented case, levels remained elevated for over a century in an area in Nevada surrounding a mercury point-source that released 7,500 tons of mercury into the environment in the 1800s (Bradford et al. 2012).

Mercury pollution is an enduring and growing problem throughout the world. While there has been some progress made in developed countries, emissions continue to rise, especially in industrializing nations of Asia, where some countries have virtually no regulations on mercury emissions (Pacyna et al. 2006). Considering human population expansion, the global energy crisis, and increasing demand for fossil fuel combustion, mercury is expected to continue increasing over the coming decades (Driscoll 2007). For example, projected increases in global temperatures are likely to increase Hg emissions through higher rates of methylation, release from melted permafrost, and more abundant forest fires (Jacob and Winner 2009). Thus anthropogenic releases of mercury will likely remain an important topic of research and public debate into the future.

iii. Mercury research at The College of William and Mary

Mercury research at The College of William and Mary began at the site of historic mercuric sulfate contamination in the headwaters of the Shenandoah River, which occurred from 1929-1950 (Carter 1977). Mercury served as a catalyst in the production of polyester at a textile company at this mercury point-source. Three decades later, high MeHg levels were found in sediment and fish just downstream of the pollution source (Carter 1977). High levels of MeHg remain present throughout the aquatic and terrestrial ecosystem in the South River watershed more than half of a century later (Cristol et al.

2008). Belted Kingfishers (*Megasceryle alcyon*), which consume a diet of fish, have elevated levels of blood mercury at this site. More surprisingly, many terrestrial-based songbirds, such as Tree Swallows (*Tachycineta bicolor*), Carolina Wrens (*Thryothorus ludovicianus*), and House Wrens (*Troglodytes aedon*), have elevated blood mercury levels as well (Cristol et al. 2008). It is hypothesized that mercury biomagnifies in spiders, which are common in these species' diets, as many spider species consume invertebrates that emerge from the contaminated river and flood plain. The wild songbirds inhabiting mercury-contaminated areas of Virginia exhibit reduced reproductive success (Brasso and Cristol 2008), altered stress hormone profiles (Wada et al. 2009), and disrupted immune functioning (Hawley et al. 2009) compared with the same species in uncontaminated reference habitats.

Understanding sublethal effects of mercury will allow us to better determine whether there is an environmentally “safe” level of mercury. However, finding an environmental threshold that can accurately be applied across species is challenging considering the amount of variation of mercury exposure and sensitivity within and across species. The present study was designed to provide data that will begin to aid our understanding of whether relatively low levels of mercury can affect flight and molt in songbirds, which are two important processes that relate to overall fitness.

iv. Effects of mercury on birds and other wildlife

Birds are useful bioindicators of mercury pollution (Evers et al. 2005) and research on birds has played a role in forming environmental policy (Driscoll et al. 2007). Mercury levels in bird feathers and tissues have been measured extensively (Altmeyer et al. 1991;

Burger and Gochfeld 1992, 1997; DesGranges et al. 1998; Thompson et al. 1998; Rimmer et al. 2005; Burger and Eichhorst 2007), but relatively little research has attempted to assess the effects of mercury on the health and fitness of birds. The limited number of studies that have measured sublethal effects of mercury in birds have mainly involved aquatic species; research on terrestrially foraging birds lags far behind (Seewagen 2010).

Methylmercury is most recognized for its neurotoxic effects that have been demonstrated across many taxa. It is known to cause disturbances in myriad neurological endpoints such as neuromuscular function, motor control, memory, and cognitive ability (Cai 2011). It is a particularly powerful neurotoxin because it can cross the blood brain barrier and enter cerebrospinal fluid (Wolfe et al. 1998).

Many studies have demonstrated that mercury affects a wide array of behaviors that involve neuromuscular functioning, which is critical for flight. Reduced coordination, locomotor speed, and changes in responsiveness to stimuli have all been documented in birds and other taxa. For example, slower reaction time to stimulation, decreased balance, difficulty flying, and severe ataxia were documented in a captive-dosing study on Great Egrets (*Ardea alba*) fed 5 parts per million (ppm) MeHg (Spalding 2000b). Similar disturbances in locomotion were seen in Mallards (*Anas platyrhynchos*) fed 10 ppm MeHg (Hoffman & Heinz 1998). Red-tailed Hawks (*Buteo jamaicensis*) dosed with MeHg had weakened muscles and showed erratic locomotor behavior (Fimreite and Karstad 1971). A lack of locomotor coordination and changes in sensory response has been positively correlated with mercury exposure in birds (Finley et al. 1979; Laties and Evans 1980; Bouten et al. 1999), fish (Alvarez et al. 2006; Jakka et al.

2007). and amphibians (Burke et al. 2010). Generally, the effects observed across taxa involve decreased locomotion performance and exaggerated reaction to stimuli.

Considering the comprehensive effects of mercury on locomotion and response behavior, it is reasonable to assume that birds consuming mercury may suffer deficiencies in escape take-flight performance.

The mechanisms underlying the neuromuscular changes discussed above have not been fully elucidated. It is likely, however, that oxidative stress plays a role (Manfroi et al. 2004; Aschner et al. 2007; Glaser et al. 2010). Oxidative stress occurs when a biological system cannot detoxify reactive intermediates within the cell or repair the damage that the imbalance of reactive species causes. Glaser et al. (2010) found that mercury disrupts creatine kinase activity, which leads to oxidation of thiol content in the mitochondria and subsequent oxidative stress. Creatine kinase is an essential enzyme for cellular energy homeostasis and is required by tissues that consume energy rapidly, such as cardiac and muscular tissues (Glaser et al. 2010). Even low levels of aquatic MeHg can inhibit mitochondrial production of ATP and reduce molecular respiration (Cambrier et al. 2009). And in Common Loons (*Gavia immer*) exposed to environmental mercury, this effect is thought to reduce deep-diving ability, which is critical for foraging (Olsen 2000). This effect of mercury on bioenergetics could cause the deficiencies in neuromuscular function and locomotion that have been observed across taxa.

In addition to mercury's neurotoxic and bioenergetic effects, it also causes behavioral changes in birds living in contaminated regions or experimentally dosed with mercury, ranging from altered parent/offspring interactions to changes in reproductive and self-maintenance behaviors. For example Mallards (*Anas platyrhynchos*) dosed with

0.5 ppm MeHg laid more eggs outside the nest box and the young of these birds did not react to threatening stimuli or respond to their mother's calls appropriately (Heinz 1979). Also, environmentally-exposed adult Common Loons (*Gavia immer*) had a lower frequency of feather preening and carrying offspring on their backs (Nocera and Taylor 1998). In another study of free-living loons, attentive nest behavior and foraging were less frequent in birds that had more than 3.0 ppm wet weight MeHg in their blood (Evers et al. 2008). Courtship and mating behavior has also been studied in relation to mercury. An increase in same-sex pairing and reduced courtship behavior was reported in male White Ibises (*Eudocimus albus*) dosed with 0.05 ppm – 0.3 ppm wet weight MeHg (Frederick & Jayasena 2011).

Several interrelated mechanisms likely underlie mercury's effect on behavior. For example, oxidative stress and reduced bioenergetics (discussed previously) could cause general lethargy, which would affect an array of behavioral endpoints. Another possible explanation for behavioral changes is that mercury causes reduced GABAergic and glutamatergic neurotransmission, both of which are critical neurological pathways involved in vertebrate behavior. Receptors in these pathways have decreased neurological activity in the brains of free-living Bald Eagles (*Haliaeetus leucocephalus*) exposed to dietary mercury (Nam-Dong Ha et al. 2012).

v. Rationale for Studying the Effects of Mercury on Flight

Mercury is a widespread and persistent environmental contaminant that occurs in aquatic and terrestrial habitats. Songbirds that forage from primarily terrestrial sources have recently been demonstrated to bioaccumulate mercury. There are many indications that

mercury negatively affects neurological functioning, bioenergetics, and behavior in a wide array of avian taxa and through a variety of mechanisms. This suggests the potential for an effect on flight performance, since flight performance requires efficient neurological, energetic, and behavioral functioning. Mercury could also affect many of the physiological mechanisms of flight, such as the functionality of feathers and wings. For example, molt (the process by which feathers are replaced annually) affects flight by reducing overall wing area (Swaddle and Witter 1997). In addition, the rate of molt affects feather quality (Dawson et al. 2000), which can influence flight performance in European Starlings (*Sturnus vulgaris*) (Swaddle et al. 1996). Hence, in this study I have investigated the influence of mercury on measures of flight performance and also molt. Flight is perhaps the most critical behavior in a songbird's life and thus any mercury-induced alterations in flight performance or molt, even if they are slight, may reduce fitness.

Section II: Flight

i. Flight performance

In an ecological context, the term “performance” is defined as the proficiency by which an organism executes a behavior (Burke et al. 2010). For birds, flight is arguably the most important behavior to perform well because it relates to virtually all other activities in a bird's life. For example, decreased flight performance could influence individual fitness through reduced proficiency at escaping predators (Lima 1993; Witter et al. 1994), provisioning offspring, migrating seasonally, or competing for food. Exposure to toxins

can promote expression or suppression of certain behaviors and alter performance (Henry and Atchison 1991), so it is important to understand whether a common pollutant such as mercury impairs this behavior.

Flight behavior differs depending on the motivation of the bird as well as the ecology and evolutionary history of a species (Van den Hout et al. 2010). There are various modes of flight such as take-off flight, forward flapping flight, flap-bounding, flap-gliding, and soaring (Gill 2007). Many birds employ a combination of flight modes depending on their evolutionary history and behavioral or ecological context. Escape take-off and maneuverability during forward flapping flight are important flight behaviors for European Starlings (here after referred to as “starlings”). Starlings are insectivorous ground foragers (Feare 1984), so escape take-off flight is critical for evading terrestrial predators and some ambushing raptors. Maneuverability during forward flapping flight is also an important flight tactic (Gillies et al. 2011) and is commonly employed by flocks of starlings to evade aerial predators. The present study examines the effects of sublethal levels of mercury on take-off flight and maneuverability because of their relevance to predator escape in starlings, which are a model organism for flight performance (Rayner 1985; Fryday et al. 1995; Swaddle et al. 1999; Tobalske and Dial 2000; Dial 2008). Escape take-off and maneuverability are especially important to study in the context of mercury because they require a large amount of energy, efficient sensory conduction, and appropriate neuromuscular coordination—all of which are known to be affected by mercury.

ii. Flight energetics

Flight is arguably the most energetically expensive activity that a bird engages in, and different modes and speeds of flight require different amounts of energy (Gill 2007). The present study measures take-off flight, which requires a relatively large amount of energy (Marden 1994) and maneuverability, which requires efficient neuromuscular function to achieve dexterity (Dudley 2002).

Theoretical modeling based on kinematics and morphological data has amounted to the fixed-wing aerodynamic theory, which posits a “U” shaped power curve for level flapping flight (Pennycuick 1968, 1997). This means that birds use the most power at the lowest and highest speeds and best conserve energy at intermediate speed—the speed at which they fly during sustained flight. Sustained flight during long-distance travel is necessary for migratory birds and is maintained by aerobic metabolism of fuel substrates. In the present study, maneuverability during forward flapping flight was measured while the birds were flying at low speeds, and thus using a relatively large amount of energy. Take-off requires that a bird generates enough airflow across the wing to create lift from a standstill, thus it is also energetically expensive. Unlike sustained flight, which is largely aerobic, birds engaged in take-off are in a state of anaerobic metabolism and thus theoretically use the maximum amount of energy possible (Marden 1994). Anaerobic respiration results in excess build-up of lactic acid, metabolic acidosis, and oxidative stress (Burns et al. 2010), so birds must recover after an escape flight (Bishop and Butler 2000). Both the take-off performance and subsequent recovery could be affected by Hg contamination.

One of the most commonly studied physiological variables of flight is the rate of biological energy expenditure, which is also called “power input” (P_i). Mechanical power available for flight comes directly from the pectoralis and supracoracoideus flight muscles. The pectoralis muscle is responsible for 95% of the power used during flight (Biewener et al. 1992). This pair of muscles undergoes sequential lengthening and shortening contraction to power flight. The muscle lengthens during the upstroke, which creates a force. Then, energy is expended during the down stroke as the muscle shortens. This change in muscle length (along with jumping force from the legs) generates lift and thrust to overcome wing inertia and initiate flight. Available chemical energy comes from catabolism (anaerobic or aerobic) of molecules that are transformed by the myofibrillar proteins of the flight muscles into mechanical power output (P_o) and heat (Butler & Bishop 2000). Therefore, P_i is dependent on the efficiency of P_o . The unit used for power is the watt (W), which is equal to 1 joule (J) per second.

iii. Take-off flight

Take-off escape has become an active field of research due to its importance in predator-prey interactions (Swaddle and Lockwood 2003; Williams and Swaddle 2003; Renner 2006). There are many different ways to measure take-off and it is often unclear which is the most suitable for the question being posed (e.g. time to cover a particular distance, velocity, maximum acceleration, mechanical energy expended, power output, etc.). When interested in maximum performance ability, it may be useful to compare peak acceleration during take-off (Renner 2006). In Least Auklets (*Aethia pusilla*) peak power (W) and peak acceleration (m/s^{-1}) occurred at about 0.4 seconds after take-off and this

specific time period was the most repeatable (Renner 2006). This measurement is useful in assessing maximum performance ability, however, in studies that attempt to assess efficacy of escaping an actual predation event, maximum acceleration may not be the most relevant metric. In an ecological setting, a multitude of other factors are important. For example, when a bird is attacked it has to make rapid decisions about the angle and speed of take-off because there is a trade-off between linear acceleration and climb rate (Witter and Cuthill 1993). Thus, a bird that decides to take-off at a steep angle from a ground predator accelerates at a lower maximum than it would at a shallower angle. Swaddle et al. (1999) employed a metric that summarizes the trade-offs between height gain and speed gain by calculating joules of energy expended with height, mass, and velocity as variables of the equation. I employed this metric in the present study because its attention to the trade-offs between speed and angle make it more applicable to escape take-off in ecological settings, where birds must decide what the most efficient escape method is depending on the predator.

iv. Maneuverability

Maneuverability is defined by the radial size of a turn; the smaller the radius, the better the turn (Warrick 2002). Animal maneuverability flight is achieved as accelerations and directional changes, which require dexterity and precision in flight (Dudley 2002). Maneuverability in the air is critical for many predatory species as well as species that need to avoid predation (Hedenström and Rosen 2002). Dodging predators at the last moment is a common predator escape strategy for birds, especially starlings (Lind et al. 2002). This tactic is common in passerine species because it requires relatively slow

speeds and low body mass compared to predators, which are often heavier and flying at faster speeds as they attack (Howland 1974). Quick turns and erratic flight by birds in flocks is especially useful for open-country species such as starlings because this behavior can confuse and startle predators (Caro et al. 2004).

Maneuverability is related to fitness because slight effects on coordination during turning flight may increase the risk of collision and make a bird more vulnerable to predation (Hunt 1992). But maneuverability is not well studied in birds (Gillies 2011) and many efforts to quantify maneuverability have underlying motivations related to comparative phylogenetics and functional morphology rather than performance (e.g. Hedenström and Rosen 2001; Matyjasiak et al. 2004; Dial et al. 2008; Jackson and Dial 2011). While methods applied in these types of studies are useful in their own fields, many of them would not be able to measure actual performance or detect fine differences in performance that occur between individuals of one species. A limited number of studies have aimed to measure intraspecific maneuverability performance by testing obstacle avoidance or overall time to navigate through an obstacle course (Fryday et al. 1995; Swaddle and Lockwood 2003; Matyjasiak et al. 2004). I chose to examine speed and angle of maneuverability because these are more precise measurements of turning flight and starlings are known to rely on quick turns while evading predators. To my knowledge, this is the first study that aims to assess maneuverability by measuring actual speed and angle while birds perform a single turn around a barrier during forward flapping flight.

It is important to point out the difference between *agility* and *maneuverability*, which were first defined by Norberg and Rayner (1987) in an extensive analysis.

Maneuverability involves taking tight turns at the shortest possible radius and requires low wing loading (low aspect ratio), whereas agility refers to the quickness of change in speed and direction. Low aspect ratio, while beneficial to maneuverability, can actually impede agility, meaning that efficient turning maneuverability (minimum radius length) requires flying at relatively slow speeds (Dudley et al. 2002). Recently, turning maneuverability was distinguished from linear maneuverability (Warrick 1998). Turning maneuverability involves changing the direction of velocity; and linear maneuverability involves the magnitude of change in velocity (acceleration). In the present study, I measure overall angle and velocity, which generally reflect both turning (angle) and linear (velocity) maneuverability. These variables are not precise enough to reflect actual turning or linear maneuverability performance according to the above definitions, but may reflect likelihood of survival in situations that require making a turn during forward flapping flight.

v. Why mercury may affect flight performance

Because mercury has been demonstrated to affect several aspects of neurophysiology and health (Boening, 2000; Scheulhammer et al. 2007; Seewagen, 2010), it has the potential to impact a complex and demanding task such as flight. Some flight mechanisms that could likely be disrupted include efficient muscular energetics and motor sensory nerve conduction, proper feather quality, and appropriate predator response behavior—all of which are essential for proficient flight (Metcalf and Ure 1995; Swaddle et al. 1996; Lockwood et al. 1998; Swaddle et al. 1999; Stevenson 2000; Tobalske and Dial 2000; Tobalske et al. 2005). Decreased bioenergetic capability in relation to elevated mercury could also affect flight performance by causing inefficient muscular output or inability to

maintain energy-expensive activities for long durations of time (Cambrier et al. 2009; Glaser 2010). Disruptions of any of these non-mutually-exclusive mechanisms may affect distinctive parameters of flight performance.

Section III: Molt

i. Bioenergetics of molt

Molt is closely related to flight and has been shown to affect take-off flight in starlings specifically. Swaddle and Witter (1997) found that natural and simulated molt reduces take-off and causes changes in anti-predator tactics. Subsequently, Swaddle et al. (1999) demonstrated that energy exerted during the second wing beat was responsible for slower escape take-off in starlings in a state of simulated molt. Molt creates gaps in the flight feathers as feathers are lost and regrown, so it may be hypothesized that the observed reductions in flight performance were a result of decreased wing area. Because molt is so closely tied to flight performance, it was important to monitor this process during the present study. In the following subsections I will discuss the mechanisms of molt and propose hypotheses concerning why mercury may cause changes in molt, feather quality, and thus—flight performance.

Like flight, molt has intense energy requirements. During molt, at least 25% of a bird's dry mass is lost and regenerated as feathers (Murphy and King 1992). Birds require more oxygen and increase their basal metabolic rate (BMR) by up to 46% (Walsberg 1983) during this period. The costs of molt include the biosynthetic costs of producing new feathers, heat loss due to inadequate insulation, energy intake to supply sulfur-

containing amino acids necessary for feather synthesis, and reduced flight performance (Walsberg 1983; Swaddle and Witter 1997).

ii. Circannual rhythms and molt

Molt is closely tied to circannual rhythms. For example, manipulations of the photoperiod can speed up or slow down the onset of molt in birds within controlled settings (Gwinner 1981, 1991). To date, it is not entirely clear what neurological control systems regulate circannual cycles in birds, but there is evidence that the visceral forebrain system (VFS) is intimately involved (Kuenzel 2000). The VFS may orchestrate circannual rhythms through fluctuations in sympathetic versus peripheral nervous system dominance during different periods in the annual cycle. For example, from the beginning of spring migration to the beginning of pre-alternate (spring) molt, the sympathetic nervous system (SNS) dominates. The SNS is responsible for loss of body mass and lipid reserves, which are necessary during migration. When birds begin to molt in the spring, there is a shift from SNS dominance to peripheral nervous system (PNS) dominance. The PNS is responsible for provoking a photorefractory state, which means birds are no longer responsive to photoperiodic changes (Kuenzel 2000). When this change occurs, birds no longer display migratory behavior, but instead begin physiological changes that are conducive to reproduction and parental care (Kuenzel and Helms 1974). Thus, the timing of molt is closely tied with nervous system changes that regulate other major circannual life history events such as migration and reproduction.

The precise biological mechanisms behind molt are somewhat unclear, but it is recognized that hormonal fluctuations play a major role. For example, thyroid hormones

are elevated in blood plasma during molt (Brake et al.1979; Lien and Siopes 1989) and injecting thyroid hormones can induce molt in captive birds (Verheyen et al. 1983; Sekimoto et al. 1987). In starlings specifically, testosterone suppresses onset of molt and also decreases the rate of molt (Shleussner et al. 1985). Large amounts of thyroid hormone have anti-gonadal effects, so when thyroid hormone is high, birds cease reproductive activity and begin pre-basic molt in the fall. Decreased estrogen is also linked with molt (McNabb 2000), and a high thyroid hormone/estrogen ratio may be important in forming new feathers (reviewed in, Decuypere and Verheyen 1986).

iii. Why and how mercury may affect molt

It is well known that mercury accumulates in feathers, especially during molt (Swiergosz 1998; Thompson et al. 1998; Condon and Cristol 2009). This occurs because mercury binds to sulfhydryl groups (Crespo-Lopez et al 2009), so disulfide-rich keratin proteins in growing feathers make them a prime pathway for mercury excretion (Eisler 2006). Since mercury is deposited directly into the feathers, the structural architecture and integrity of the feathers could be affected. Weak feather structure (for example smaller diameter in feather rachis or larger spaces between barbules) can cause increased feather abrasion and decreased resistance to external parasites (Vagasi 2011).

Disruption of the production of corticosteroids, estradiol, and testosterone have been linked to mercury exposure (Hontela et al.1992; Giesy 2003; Heath & Frederick 2005; Frederick & Jayasena 2011). Wada et al. (2009) found a negative relationship between blood mercury and thyroid hormone in free-living Tree Swallows (*Tachycineta bicolor*), a passerine bird. The interaction between thyroid hormones and sex-steroids can

affect hormonal regulation of molt (Hahn 1992), hence changes in any of these hormones could lead to changes in the timing and pattern of molt. Like flight, molt is also energetically expensive (Murphy and King 1992) so any disruption in bioenergetics caused by mercury may also affect molt.

In the present study, I predicted an increased rate of molt. Evidence shows that birds under physiological stress or with less access to reliable food sources molt at a faster rate. For example, birds that live in habitats with plentiful food sources year round have more prolonged molt than birds inhabiting areas where food is only available for short periods of time or where there is large variation in climate (Hahn 1992). Thus, any perceived decreases in energy intake caused by mercury may cause increased molt rate. The known effect of mercury on testosterone also suggests that dosed birds may molt more rapidly. As discussed, mercury is known to cause decreases in testosterone and lowered testosterone levels cause earlier and faster molt.

Section IV: Experimental Approach

i. Model organism

Starlings are a common species (Order: Passeriformes; Family: Sturnidae) species that occurs in large numbers on almost all continents (Feare 1999). Starlings are thought to be native to most of temperate Europe and parts of Eastern Asia. They are resident (non-migratory) in their native lands yet partially migratory in areas where they have been introduced, which include Australia, North and South America, Africa, and New Zealand.

Starlings are highly social, migrating in flocks as big as tens of thousands. They fly well, commonly at speeds around to 18 ms^{-1} (Feare 1999) and are known for their accuracy and dexterity as they evade aerial predators in large flocks or “murmurations”. Their flying behavior involves complex social coordination and locomotor acuity (Lima 1994).

Starlings have been used in detailed studies of the functioning of flight musculature and the mechanics of flight (Goslow et al. 1987; Jenkins et al. 1988; Biewener et al. 1992; Swaddle & Biewener 2000; Dial et al. 2008). They have a P_i of about 9W during sustained flap-gliding flight, which allows them to maintain the optimal wing-beat frequency for energy efficiency (Rayner 1985).

ii. Research questions

The purpose of my study was to determine whether environmentally relevant levels of chronic dietary mercury are deleterious to flight performance and/or molt. I was interested in three major questions regarding sub-lethal mercury exposure: (1) Does mercury affect molt patterns? (2) Does mercury affect flight performance (take-off and maneuverability)? (3) Is there an interaction between molt and other effects of mercury on flight?

To answer these questions I measured within-individual changes in flight performance and differences in molt in captive starlings that were dosed with chronic, sub-lethal levels of mercury over the course of one year. I measured flight performance in two assays that tested the following variables: (1) Take-off angle and speed combined into a metric of energy gained during flight in response to a predator stimulus (Swaddle et al. 1999); and (2) velocity and angle while maneuvering around an obstacle in the

flight path to escape a predator stimulus. In addition, I performed an assessment of molt timing and pattern (Ginn and Melville 1983) and compared metrics of molt between the mercury exposure treatment groups.

Flight is a complicated and energetically demanding adaptation in birds, and involves virtually every physiological system in some way. Considering the array of negative health effects of MeHg in birds, I hypothesized that disruptions of several physiological, neurological, and behavioral mechanisms of flight would lead to reduced energy expenditure during take-off and reduced velocity during maneuverability in mercury-dosed birds. I also hypothesized that disruptions of normal hormonal fluxes would increase molt rate between the groups and subsequently would make any differences in flight performance between the groups more pronounced.

iii. Significance of research questions

It is necessary to experimentally test MeHg effects at the organismal level to assess whether mercury poses a risk to passerine survival and population sustainability. While reduced reproductive success is commonly associated with mercury toxicity, the mechanisms of such change are unknown. I hypothesized that the many effects of mercury on neurological functioning, behavior, and the endocrine system would lead to decreased flight performance and increased molt rate. Small deficiencies in flight are difficult to measure without a controlled environment and, as far as I know, there have not been any studies of flight performance in birds dosed with mercury.

Flight is a suitable measure of fitness because virtually all activities in a passerine bird's life involve flying: foraging, mating behavior, parental care, predator escape, and

migration are some examples. Migration is one of the most important components of passerine survival and is remarkably understudied in relation to mercury (Seewagen 2010). In fact, 85% of adult mortality of passerine birds may occur during migration (Sillet and Holmes 2002). Deficiencies in flight performance may or may not apply directly to migratory efficiency, but my data could prompt further research and provide some insight. Overall, decreased flight ability will likely directly affect survival probabilities in a small bird such as a starling.

Flight and migration efficiency in passerines is one area that is recognized as a gap in ornithological research (Seewagen 2010). Mercury is a ubiquitous pollutant with known deleterious effects on birds. Thus, it is very important to determine whether mercury affects flight performance. With enough data on passerine receptors of environmentally relevant levels of mercury, we can help predict what level of environmental mercury exposure causes harm and thus the degree to which emissions reduction is necessary to safeguard songbirds.

METHODS

Section I: Animal Housing and Dosing

I performed this study on hatch-year (HY) starlings captured from the wild during May-July 2010. The birds were housed in groups of 8-10 in large outdoor cages (2.5Wx2.5Hx3.5L m) and given food (Bartlett poultry starter crumbs) and fresh water *ad libitum*. Cages were consistent in shape, volume, and perches (Ashler 2009). I rotated groups through the cages and reshuffled the birds within their treatment groups every six weeks to control for differences in social environment.

Birds were dosed (n = 20 in each treatment group) with methylmercury-cysteine (MeHgCys) through consistent dietary intake at 0.0 parts per million (ppm), 1.5 ppm, or 3.0 ppm wet weight (equivalent to 0.00 ppm, 1.83 ppm or 3.66 ppm dry weight) beginning in March 2011. Within six weeks on this treatment, three of 20 birds in the highest dosed group died. The determined cause of death was kidney failure, which was based on glomerular filtration rate (GFR) tests. The treatments were thus reduced to 0.75 ppm and 1.5 ppm (wet weight, equivalent to 0.92 ppm and 1.83 ppm dry weight) respectively since the purpose of the study was to measure sublethal effects of mercury exposure. There was no further mortality or obvious detrimental effects on general health due to mercury in either group throughout the study. Post-dosage flight tests had not yet commenced when I adjusted the doses; so all results presented here are from birds that were on the second dietary regime.

All blood and food measurements were performed using a Milestone Direct Mercury Analyzer (DMA-80). Blood samples were collected every month in capillary tubes by puncturing the brachial vein with a 34-gage needle. Capillary tubes were placed in labeled vacutainers and kept in a freezer until analysis in the DMA. Between 90% and 100% of mercury in avian blood and feathers is MeHg (Rimmer et al. 2005; Wada et al.

2009). so it is safe to assume that the measured Hg levels are closely correlated with MeHg levels.

Section II: Take-off Flight

I measured escape take-off in a large outdoor aviary (3Wx2.5Hx9L m) by placing birds on a perch 10 cm from the ground and sounding a whistle as the bird was released to represent an auditory predator stimulus (Fig. 1). I filmed flight at a perpendicular angle as the birds flew away from the perch across a grid (Swaddle et al. 1999). Criteria for a successful trial required birds to fly directly away from the predator stimulus for at least 6 m with no detectable fluctuation in z-axis movement. In cases where these criteria were not met in the initial release, the birds were given two additional attempts to meet criteria with a 5-min break in a small cage between releases. One flight was measured per bird per session because previous studies have demonstrated that take-off performance is highly repeatable within individuals (Veasey et al. 1998; Criscuolo et al. 2011) and that successive releases cause fatigue, which results in increasingly lower performance (Renner 2005).

I assessed three parameters of take-off flight in this test: (1) Joules of energy exerted per unit mass over the first 10 frames of take-off; (2) angle of take-off (in degrees); and (3) whether the bird flew past the 6 m mark before touching the ground or sides of the aviary. To obtain a measure of Joules exerted by each bird during escape take-off, I employed an equation for instantaneous mechanical energy (Eq.1; J.P. Swaddle, E.V. Williams, and J.M.V. Rayner 1999) where V_x and V_y are the horizontal and vertical

components of flight speed, g is acceleration due to gravity, and z is height. I then divided change in instantaneous energy by mass (kg) to obtain a measure of Joules per unit mass.

$$(1) \quad E = \frac{1}{2}[V_x^2 + V_y^2] + gz$$

As subtle changes in wing loading, asymmetry, wingtip shape, and body mass affect take-off flight (Swaddle 1997; Swaddle and Lockwood 2003; Alerstam *et al.* 2007), I attempted to minimize variation in these metrics by (a) using each individual as its own control in a repeated-measures design; (b) explicitly including body mass in the calculation of instantaneous energy; and (c) examining whether an index of feather wear related to energy exerted during takeoff. I classified feather wear based on criteria from the Monitoring Avian Productivity and Survivorship (MAPS) program in session 1 and found that feather wear score was not related to Joules of energy exerted during take-off (*Linear Regression*, $F_{1,56} = 0.0860$, $P = 0.756$) or angle of take-off (*Linear Regression*, $F_{1,56} = 0.171$, $P = 0.680$). Thus, I ignored differences in individual feather wear score in further analyses.

I performed the take-off test on five separate occasions (summarized in Table 1). The overall purpose of the repeated testing was to address whether individual changes in take-off flight over time varied across treatment groups. I was interested in assessing flight changes during two differing periods of a bird's annual cycle: (1) The non-molting period (October-May) and; (2) the molt period (May-September). To assess whether mercury had an effect on escape take-off during non-molting months I compared changes in individual flight performance across treatment groups using data from sessions 1 (pre-

dosing as an individual ‘control’ measurement), 4 (approximately 12 weeks after molt), and 5 (approximately 20 weeks after molt). To answer the question of whether mercury affected flight performance during molt I compared changes in individual performance across treatment groups using data obtained from Sessions 1, 2 (beginning of molt), and 3 (mid-molt).

Table 1: Description of take-off recording sessions in relation to mercury dosing (weeks exposed to mercury) and molt status (stage of molt).

Session	Date	Weeks on Hg	Molt Status
1: Pre-dosing	Mar 2011	0	Non-molting
2: Start of molt	May 2011	~7	Beginning of molt
3: Mid-molt	July 2011	~14	Mid-molt
4: After molt	Dec 2011	~34	Non-molting (~12 weeks after molt)
5: After molt	Feb 2012	~42	Non-molting (~20 weeks after molt)

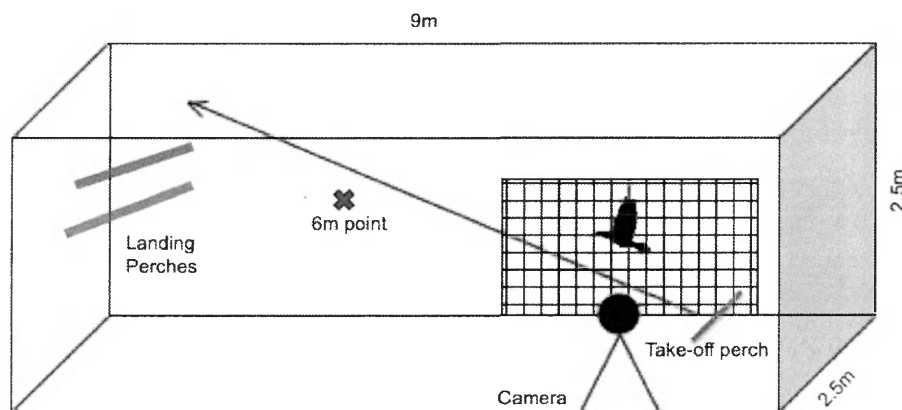


Figure 1: 3-D view of take-off experimental arena. Birds were released by hand from the take-off perch and filmed at a perpendicular angle as they flew past the 6 m point of safety to the landing perches.

Section III: Maneuverability

To measure maneuverability, I filmed birds from below as they turned around a semi-translucent screen that formed an obstacle across the width of an arena (Fig. 2). Two additional screens formed a 135° angle around the center barrier to help guide the birds around the obstacle. Birds were released by hand from a perch and filmed from below against a grid on the ceiling to allow measurement of location. The 135° angle and the opening between the barrier and the wall (50cm) were selected based on a pilot study with birds that were not used in this study.

It was necessary for the birds to learn to fly around the center barrier successfully; hence, before each session I exposed them to the maneuverability set-up in groups of three and forced them to fly around the screen four times each. I then gave the birds a 10-min recovery period before they were tested. Successful flight criteria required that the birds flew directly around the barrier to the perches on the other side of the aviary and that they flew at a height of at least 3.2 m (i.e. close to the grid on the ceiling and far from the camera below so that I could record the birds' location in each frame of video with sufficient precision). Each bird was given up to three attempts to accomplish one successful maneuverability flight per session (described below) and was always given a 5-min recovery period between attempts.

I measured three parameters of flight performance in maneuverability tests: (1) Total velocity (ms^{-1}) while navigating around the barrier; (2) angle (in degrees) while

navigating around the barrier, and (3) distance (cm) from the barrier as the bird turned the corner (Fig. 2).

I performed the maneuverability test on two separate occasions. Session A was before dosing and before molt had begun and session B was after the birds had been dosed with Hg for ~38 weeks and completed molt (Table 2). As with the take-off test, the pre-dosing session (session A) occurred in March just before the birds were dosed, however the single post-dosing session (session B) occurred ~38 weeks after the birds had been on mercury and after they had fully completed molt. This session took place between sessions 4 and 5 of the take-off experiment. The purpose of the maneuverability flight tests was to evaluate whether aspects of turning flight changed within individuals according to mercury treatment group; due to limited time I did not assess whether this form of flight also changed with stage of molt, as I did for the take-off test.

Table 2: Description of maneuverability recording sessions in relation to mercury dosing (weeks exposed to mercury) and molt status (stage of molt).

Session	Date	Weeks on Hg	Molt Status
A: Pre-dosing	March 2011	0w	Non-molting
B: After dosing	January 2012	~38weeks	Non-molting (~16 weeks after molt)

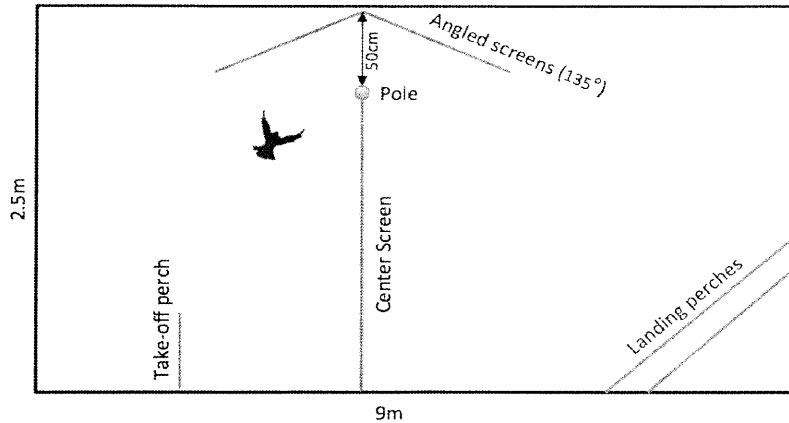


Figure 2: 2-D plan view of the maneuverability experimental arena. Birds were released by hand from the take-off perch and filmed from below as they navigated around the center screen against a grid suspended from the ceiling (not pictured) to landing perches on the other side of the aviary. Distance was measured from the first frame the bill crossed the grid to the first frame the bill exited the grid.

Section IV: Molt Assessment

I used the Ginn and Melville (1983) method to quantify molt score. This method involves assigning each of the nine primary feathers on the right wing a score from 1-5 depending on the stage of molt. For example, a score of 0 is assigned to feathers that have not been dropped while a score of 5 indicates that a new feather has fully regrown. Once each feather is assigned a number from 0-5, the numbers are summed to obtain a total molt score per wing ranging from 0-45. I took eight measurements at weekly intervals throughout the progress of molt (~100 days).

Section V: Video Analyses

I used a Sony Handycam wide-angle HDR-CX350 camera (30 frames per second at 7.1 mega pixels) to record all flight trials. I analyzed video using the public domain software program ImageJ version 1.440 (Written by Wayne Rasband at the U.S. National Institutes of Health) to digitize flight trajectories on a Macintosh OS X 10.6.8. All videos were digitized and analyzed blind to treatment groups.

Section VI: Statistical Analyses

In each of the following subsections I describe the structure of my statistical analyses and how they relate to my research questions. All statistical analyses were performed in IBM SPSS 19 (SPSS Inc. 2010) employing two-tailed tests of probability. Data are reported as means \pm 1 standard error unless otherwise noted.

i. Does within-individual change in take-off performance vary by mercury treatment group during the non-molting period (take-off sessions 1, 4, and 5)?

To test whether mercury had an effect on escape take-off during the non-molting period I analyzed within-individual changes in take-off metrics across treatment groups using data from sessions 1 (pre-dosing), 4 (12 weeks after molt), and 5 (20 weeks after molt) using repeated measures two-way ANOVA tests. Treatment (0.0, 0.75, or 1.5ppm MeHgCys) was the among-subjects independent variable and time (i.e. recording session) was the within-subjects independent variable. I further examined the effects of mercury on take-off by comparing among-group variation in the individual changes in flight metrics

between particular recording sessions. Specifically, I analyzed change in energy from session 1 to session 4 (i.e. the change from before dosing to after 12 weeks after molt while dosed) and session 1 to session 5 (i.e. the within-individual change from before dosing to 20 weeks after molt while dosed) using two separate one-way ANOVA tests. For the one-way ANOVA tests, the independent variable was treatment group and the dependent variable was difference in energy exerted by individuals. I used the same structure of analysis to examine within-individual changes in take-off angle over the same periods. The addition of these one-way ANOVA tests allowed me to increase the sample size for these research questions since the larger repeated-measures analysis included only individuals that successfully completed all three sessions. Birds that failed either session 4 or 5 were thus excluded from the repeated measures test, but could be included in one of the two one-way ANOVA tests. Where I saw notable differences in change in flight metrics among the groups, I further examined the relationships between individual blood mercury measurements and change in take-off flight metrics by employing linear regression analyses.

ii. Does within-individual change in take-off performance vary by mercury treatment group during molt (sessions 1, 2 and 3)?

To examine the within-individual changes in take-off associated with molt I performed a repeated measures two-way ANOVA using data from session 1 (before molt and before dosing), session 2 (beginning of molt), and session 3 (mid-molt). As described above, I also used two separate one-way ANOVA tests to compare differences in within-

individual change in take-off flight metrics among mercury treatments during particular recording sessions. I analyzed change from session 1 to session 2 to examine the effects of early molt; and change from session 1 to session 3 to examine the influence of mercury on flight during mid-molt. I did not examine the relationships between individual blood mercury measurements and change in take-off flight metrics (as I did for the non-molt take-off sessions described above) because there were no indications of differences among the groups during molt sessions.

iii. Do within-individual changes in maneuverability vary by mercury treatment group (sessions A and B)?

To test whether within-individual changes in maneuverability varied by mercury treatment, I performed a two-way repeated measures ANOVA test using data from session 1 (pre-dosing) and session 2 (~32 weeks post-dosing and ~16 weeks after all birds had completed molt). Unlike for the take-off test, there was no need to measure mean individual change among the groups for isolated sessions because two sessions only were included in the repeated measures analysis. I did not examine the relationships between individual blood mercury measurements and change in maneuverability flight metrics because there were no indications of differences among the groups.

iv. Does mercury treatment affect the rate of molt?

There were limitations in using the complete data set (i.e. molt scores from the entire course of molt) in my analyses because all birds complete molt with a final score of 45 hence, by definition of the metric, all individuals converge on the same molt score in later molt stages which will potentially mask among-treatment differences at other stages of molt. Thus, I used molt scores taken only during the most intensive period of molt, which occurred from June 2011-August 2011. I took molt scores on eight separate occasions during this time period (about every 10 days). During this period, all birds were experiencing a steep rate of molt and had not yet plateaued at a molt score of 45.

To examine how mercury affects the rate of molt, I analyzed within-individual changes in molt scores using a repeated measures ANOVA test and further explored differences among the groups with Tukey's post-hoc tests. The within-subjects independent variable was time (i.e. the eight occasions that molt scores were taken) and the among-subjects independent variable was treatment group. I also employed linear regression analyses to examine relationships between individual blood mercury levels and molt scores.

RESULTS

Section I: Blood Mercury Levels Throughout the Experiment

On the original mercury doses (3.0 ppm and 1.5 ppm) the higher dosed group reached a mean blood mercury peak of $14.4 \text{ ppm} \pm 1.4$ (95% CI: 11.4 ppm – 17.4 ppm) and the lower dosed group reached $7.9 \text{ ppm} \pm 0.5$ (95% CI: 6.8 ppm – 9.0 ppm). Shortly after the

Hg doses were adjusted to 1.5 ppm and 0.75 ppm the birds began to molt and deposit Hg into growing feathers (cf. Condon and Cristol 2009). During this period, the higher dosed group had a mean blood Hg level of $2.4 \text{ ppm} \pm 0.3$ (95% CI: 3.2, 6.6) and the lower dosed group $1.3 \text{ ppm} \pm 0.1$ (95% CI: 1.3, 1.4). Still on the reduced dose, after the molt period, the higher dosed group had a mean of $9.8 \text{ ppm} \pm 2.8$ (95% CI: 8.2 ppm – 11.3 ppm) blood mercury and the lower group $4.9 \text{ ppm} \pm 1.4$ (95% CI: 4.2 ppm – 5.7 ppm) (Fig. 3).

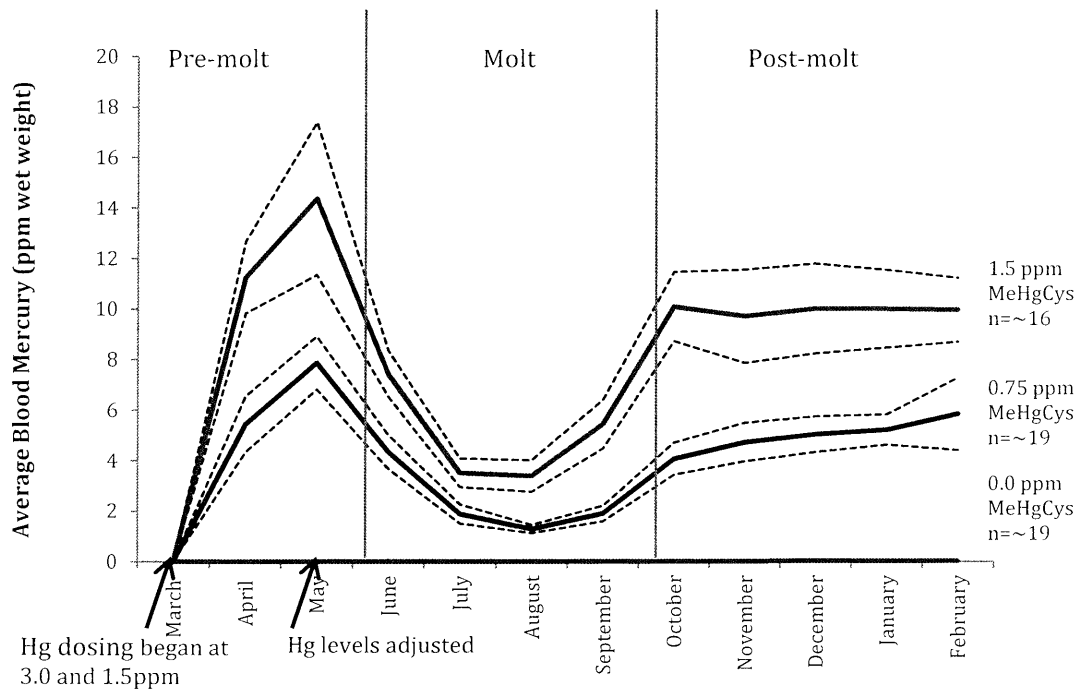


Figure 3: Mean blood mercury concentrations of birds in each treatment group throughout the experiment. Dashed lines show 95% confidence intervals.

Section II: Take-off Flight Performance

i. Does within-individual change in take-off performance vary by mercury treatment group during the non-molting period (sessions 1, 4, and 5)?

During the non-molting period, there were differences in how individuals altered energy expenditure during take-off ($F_{4,36} = 2.22$, $P = 0.076$). Specifically, birds from the 1.5 ppm treatment group started the study using more energy for take-off than control birds and lower dosed birds, but unlike the other two groups they tended to exert less energy over time (Fig. 4). Birds treated with 1.5 ppm MeHgCys exerted slightly less energy in session 4 than they did prior to dosing, while the birds in the other two treatment groups showed a gain in energy expenditure over the same time period ($F_{2,45} = 2.62$, $P = 0.076$; Fig. 5a). The same pattern was statistically significant when I compared the within-individual change in energy between sessions 1 and 5 ($F_{2,40} = 3.80$, $P = 0.031$; Fig. 5b). Birds on the control dose exerted more energy during take-off on each successive session throughout the experiment, whereas birds dosed with the higher mercury treatment exhibited a systematic decrease in energy expenditure. Birds in the lower dosed group exhibited a lesser increase in energy exertion over time, remaining about the same throughout the study (Fig. 4).

I explored these differences further by regressing individual average blood mercury during the post-molt period and change in energy exerted between sessions 1 and 4 and sessions 1 and 5. There was no consistent relationship between blood mercury and change in energy exerted between sessions 1 and 4 ($y = -0.019x + 0.144$, $F_{1,45} = 6.517$, $r^2 = 0.029$, $P = 0.160$; Fig. 6a). There was a more pronounced negative relationship between blood mercury and within-individual change in energy exerted during take-off when comparing session 1 with session 5 ($y = -0.025x + 0.249$, $F_{1,40} = 5.097$, $r^2 = 0.631$, $P = 0.030$; Fig. 6b).

Within-individual change in angle of take-off (across sessions 1, 4, and 5) varied among the mercury treatment groups ($F_{4,31} = 4.76$, $P = 0.002$). The control group as well as both mercury groups took off at a lower mean angle in sessions 4 compared to session 1 (Fig. 7a; note all three groups have a negative change in angle, indicating they flew at a lower mean angle). However, the control birds had the most pronounced decrease in angle followed by the 0.75 ppm group and finally the 1.5 ppm group, which only decreased in angle slightly compared with session 1. These differences in individual mean change in angle were statistically significant ($F_{2,45} = 10.506$, $P < 0.001$; Fig. 7a). However, the change in angle of take-off from session 1 to session 5 was not significant ($F_{2,40} = 0.350$, $P = 0.707$; Fig. 7b). The control birds and the 0.75 ppm birds exhibited a similar change in angle as they did from session 1 to session 4, however the 1.5 ppm birds flew at a lower mean angle in session 5 than they did in session 4, meaning all of the groups have a similar mean change in angle of take-off compared with session 1.

I used a Fisher's exact test to analyze whether there was a difference in the number of dosed birds compared to control birds that successfully passed the 6 m mark in each recording session. There were no differences among the groups during sessions 1, 4, or 5 ($P = 0.296$, $P = 0.312$, $P = 0.226$, respectively).

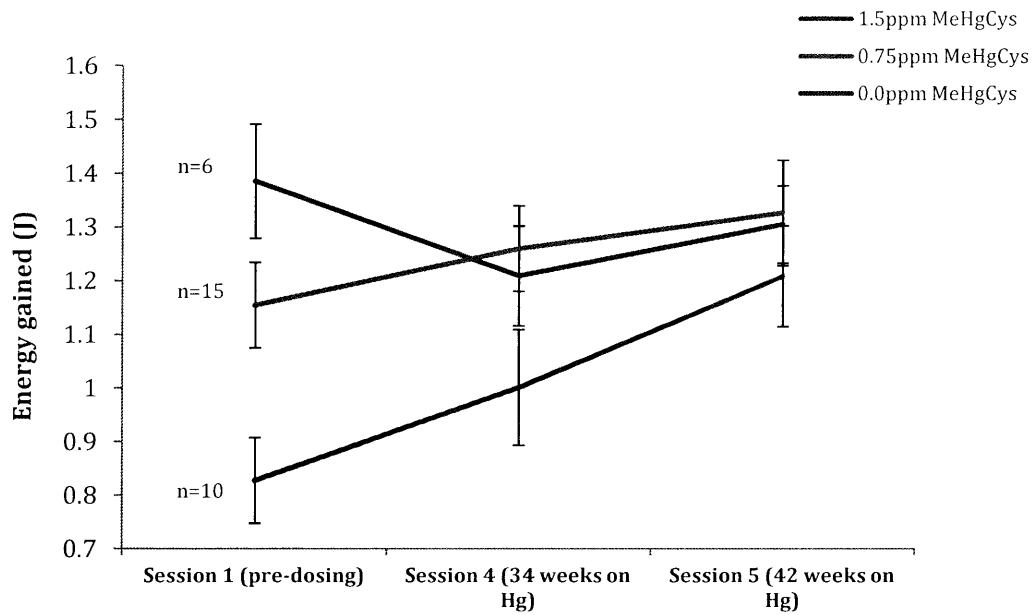


Figure 4: Mean (\pm SE) energy expended during take-off across treatment groups during session 1 (pre-dosing), session 4 (34 weeks after dosing; 12 weeks after molt), and session 5 (40 weeks after dosing; 20 weeks after molt) ($F_{4,36} = 2224$, $P = 0.076$).

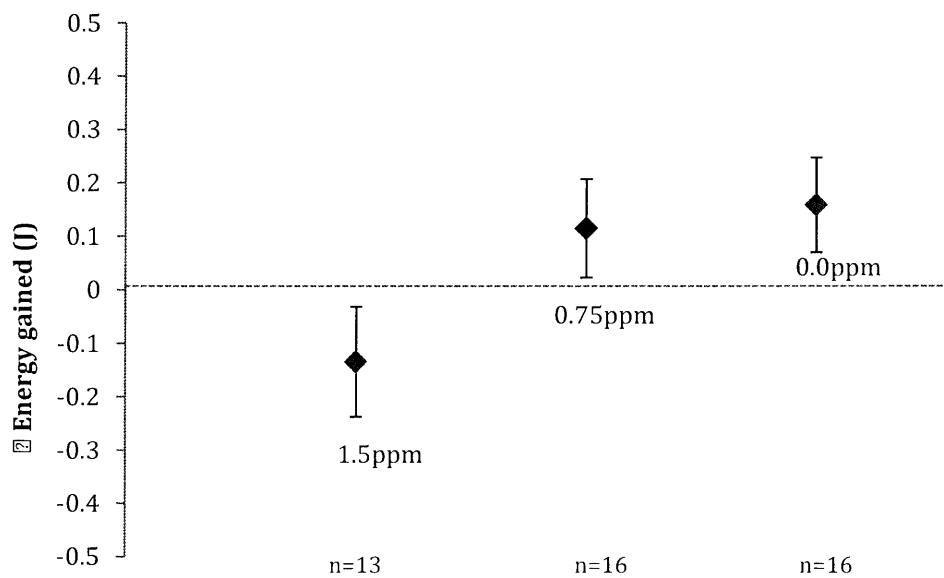


Figure 5a: Mean (\pm SE) within-individual change in energy from session 1 (pre-dosing) to session 4 (34 weeks after dosing; 12 weeks after molt) across treatment groups ($F_{2,45} = 2.62$, $P = 0.076$)

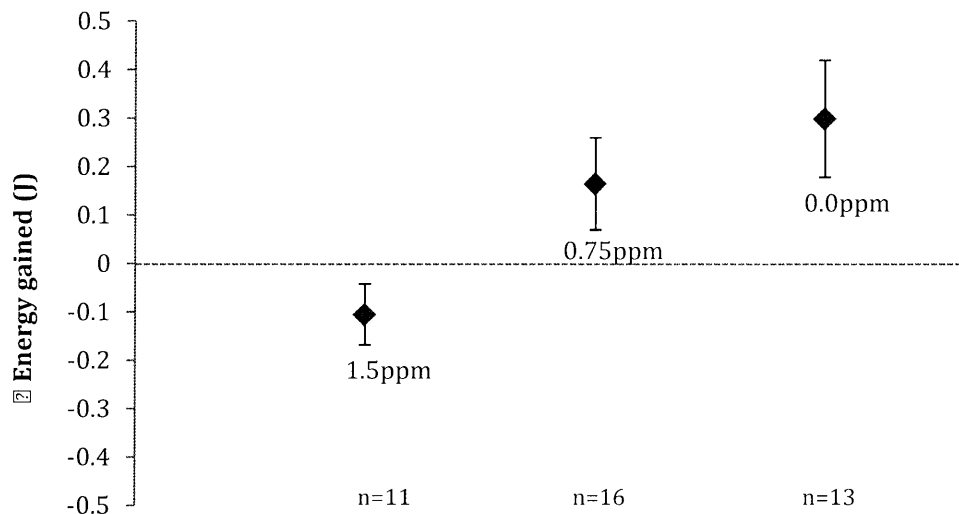


Figure 5b: Mean (\pm SE) change in energy from session 1 (pre-dosing) to session 5 (42 weeks after dosing; 20 weeks after molt) across treatment groups ($F_{2,40} = 3.80$, $P = 0.031$).

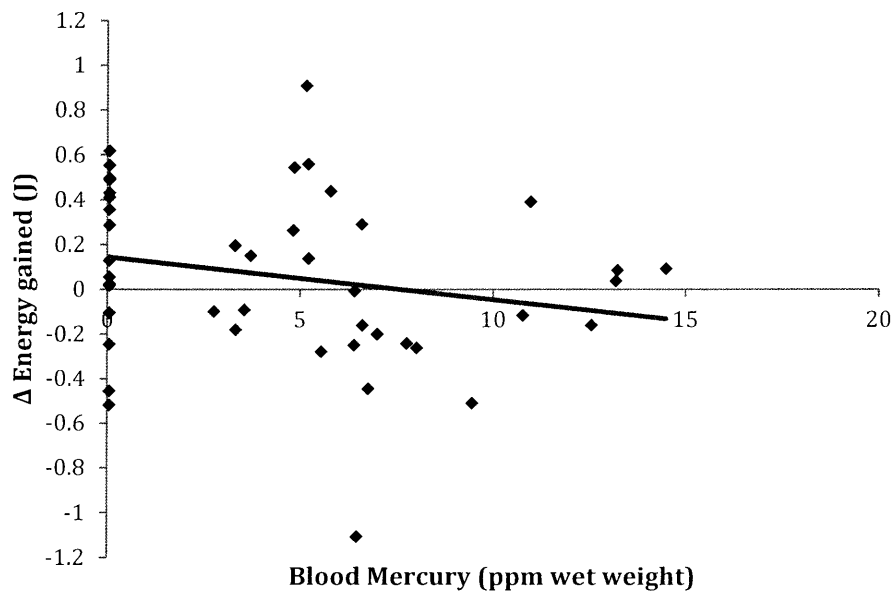


Figure 6a: Relationship between average individual blood mercury (during the post-molt period) and change in energy from session 1 (pre-dosing) to session 4 (34 weeks after molt; 12 weeks after molt) ($y = -0.019x + 0.144$, $F_{1,45} = 6.517$, $r^2 = 2.91$, $P = 0.160$).

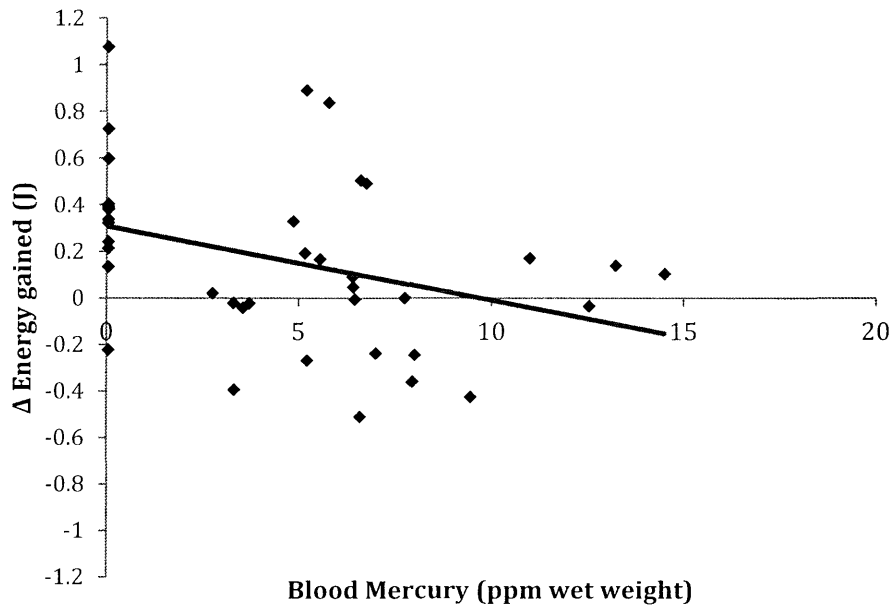


Figure 6b: Relationship between average individual blood mercury (during the post-molt period) and within-individual change in energy exerted during take-off from session 1 (pre-dosing) to session 5 (40 weeks after dosing; 20 weeks after molt) ($y = -0.025x + 0.249$, $F_{1,40} = 5.097$, $r^2 = 0.631$, $P = 0.030$).

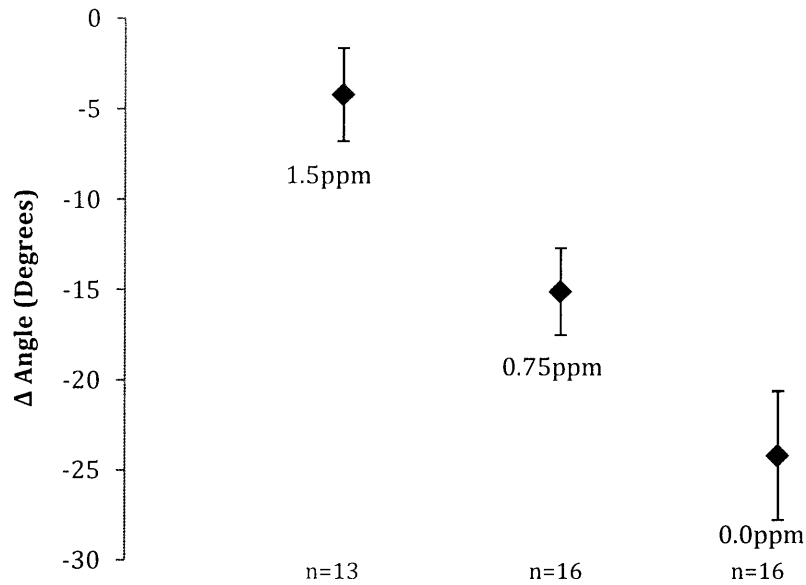


Figure 7a: Mean (\pm SE) within-individual change in angle of take-off from session 1 (pre-dosing) to session 4 (34 weeks after dosing; 12 weeks after molt) ($F_{2,45} = 10.506$, $P < 0.001$).

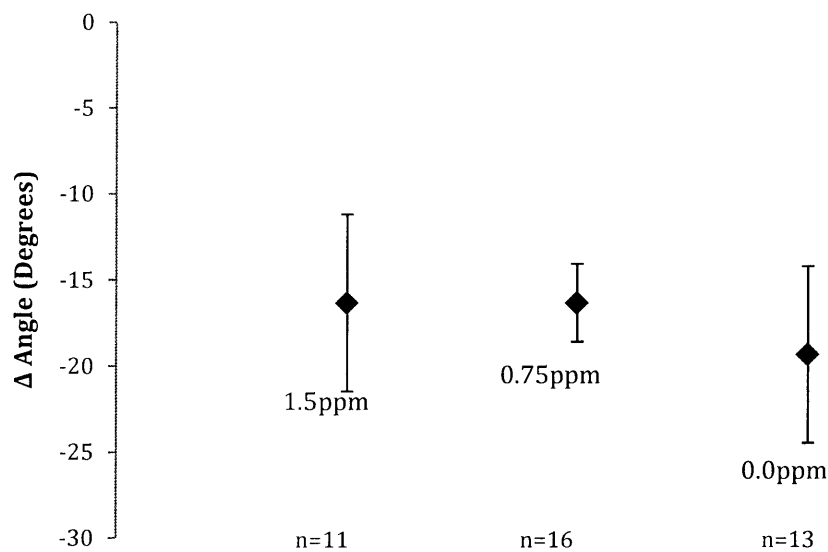


Figure 7b: Mean (\pm SE) within-individual change in angle of take-off from session 1 (pre-dosing) to session 5 (40 weeks after dosing; 20 weeks after molt) ($F_{2,40} = 0.350$, $P = 0.707$).

ii. Does within-individual change in take-off performance vary by mercury treatment group during molt (sessions 1, 2 and 3)?

While birds were molting, there were no consistent differences among mercury treatments in within-individual change in energy expended during take-off or angle of take off ($F_{2,32} = 1.08$, $P = 0.373$; Fig.8). Similarly, there were no consistent differences between the mercury treatment groups when I examined within-individual changes in energy exerted in take-off between sessions 1 and 2 ($F_{2,41} = 0.099$, $P = 0.906$; Fig.9a) nor sessions 1 and 3 ($F_{2,34} = 0.484$, $P = 0.621$; Fig.9b). Likewise, there were no differences in changes of angle of take-off from session 1 to 2 or session 1 to 3 among the treatments ($F_{2,41} = 0.981$, $P = 0.553$; $F_{2,34} = 1.27$, $P = 0.272$).

There were no differences between mercury-dosed birds and controls in the probability of passing the 6 m point during recording session 2 or session 3 (Fisher's exact test: $P = 0.368$, $P = 0.208$, respectively).

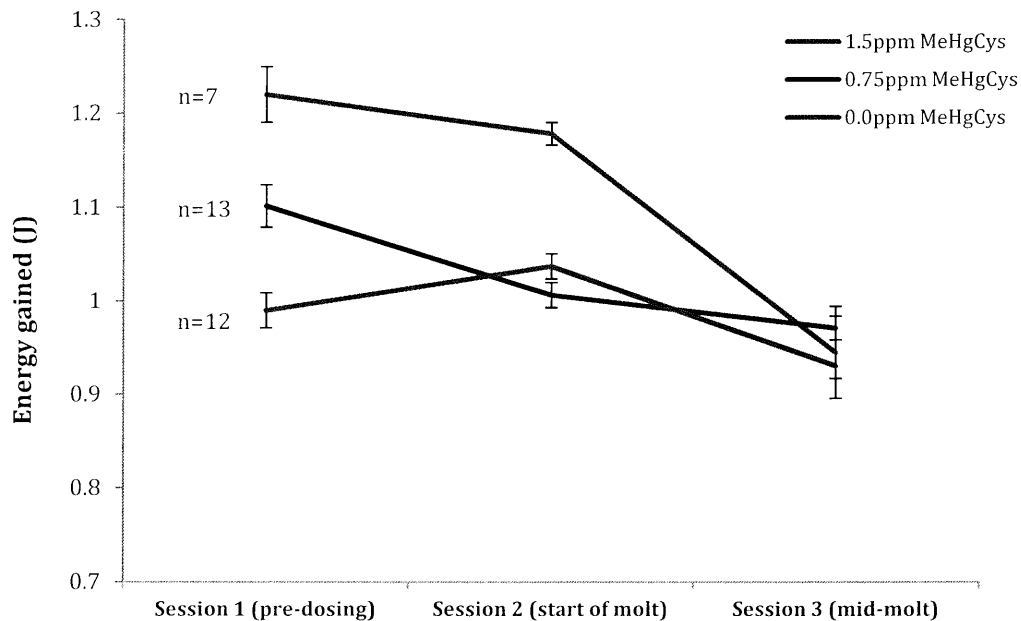


Figure 8: Mean (\pm SE) energy expended during take-off across treatment groups during session 1 (pre-dosing and pre-molt), session 2 (7 week after dosing, start of molt), and session 3 (14 weeks after dosing; mid molt) ($F_{2,32} = 1.08$, $P = 0.373$).

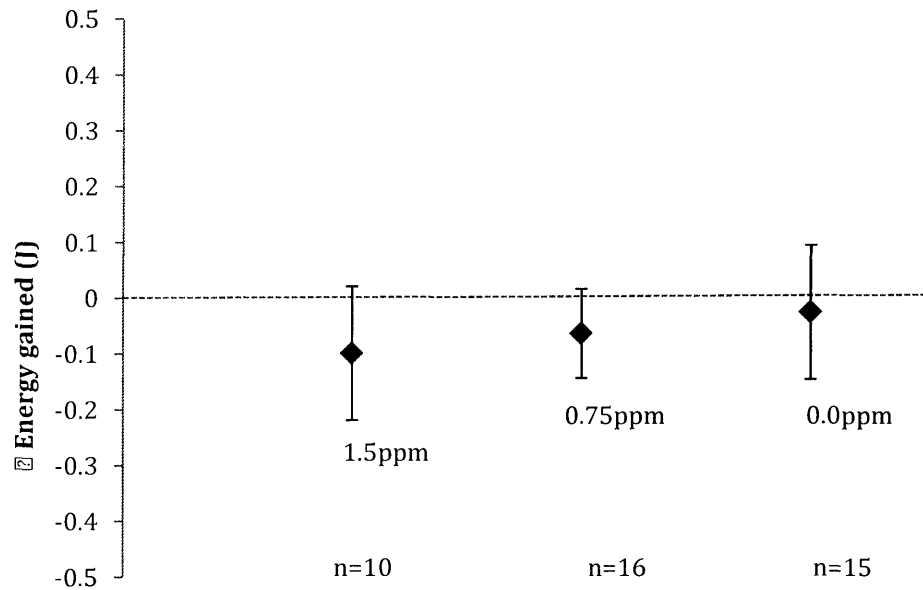


Figure 9a: Mean (\pm SE) within-individual change in energy from session 1 (pre-dosing and pre-molt) to session 2 (7 weeks after dosing; start of molt) across treatment groups ($F_{2,41} = 0.099$, $P = 0.906$).

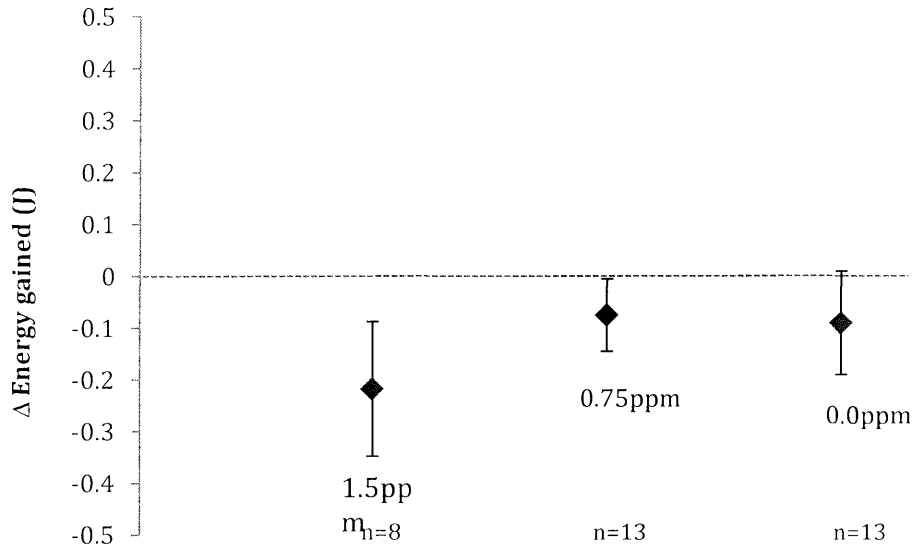


Figure 9b: Mean (\pm SE) within-individual change in energy from session 1 (pre-dosing) to session 3 (12 weeks after dosing; mid-molt) across treatment groups ($F_{2,34} = 0.484$, $P = 0.621$).

Section III: Maneuverability

i. Do within-individual changes in maneuverability vary by mercury treatment group (sessions A and B)?

To examine whether mercury had an effect on flight maneuverability, I compared results from maneuverability session A (pre-dosing) with those from maneuverability session B (38 weeks after dosing, 16 weeks after molt). Mercury had no consistent effect on velocity (ms^{-1}) or angle of turn while birds navigated through the maneuverability course ($F_{2,41} = 0.446$, $P = 0.644$; $F_{2,41} = 1.33$, $P = 0.277$, respectively). There was some indication that the change in distance from the bird to the barrier was different between groups after they had been treated with mercury ($F_{2,41} = 2.43$, $P = 0.119$). Birds treated with 1.5 and

0.75 ppm MeHgCys tended to fly closer to the barrier after being treated, while control birds tended to increase their distance from the barrier on the later tests ($F_{2,41} = 2.43$, $P = 0.119$; Fig. 10).

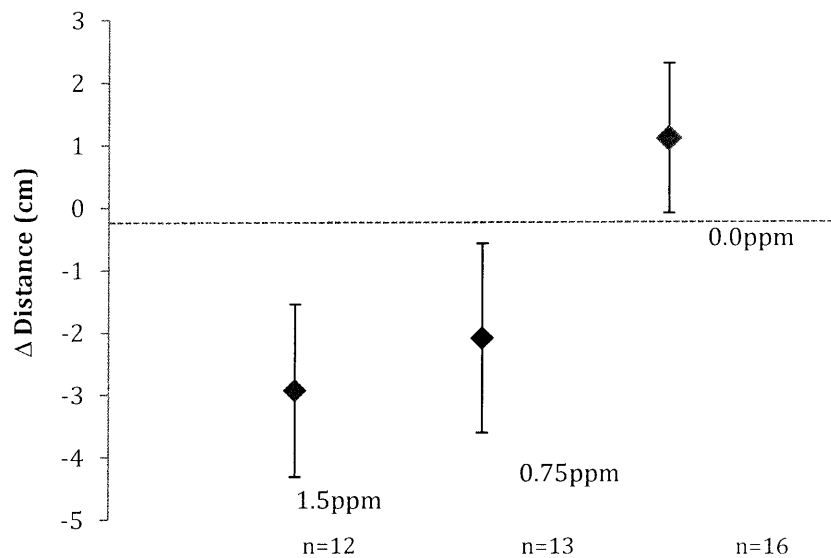


Figure 10: Mean (\pm SE) within-individual change in distance (cm) to the barrier from maneuverability session A (pre-dosing) to session B (38 weeks after dosing; 16 weeks after molt) ($F_{2,41} = 2.43$, $P = 0.119$).

Section IV: Molt Assessment

There was a tendency for birds from the highest mercury treatment to proceed more quickly through molt than the control group ($F_{2,54} = 2.46$, $P = 0.096$; *Tukey's post test*, $P = 0.09$; Fig. 11). To explore these differences in more detail, I analyzed the relationship between individual blood mercury at the onset of molt and average change in molt score per scoring occasion (May – August 2011) in individuals across the treatment groups (i.e.

a measure of the rate of molt). I found that blood mercury at the onset of molt was positively related to rate of molt ($y = 0.035x + 5.884$, $F_{2,55} = 10.133$, $r^2 = 0.16$, $P = 0.003$; Fig. 12). Thus, birds with higher blood mercury molted more quickly. I also tested for a correlation between individual blood mercury at the onset of molt and average molt score and found a significant positive relationship ($y = 0.143x + 26.845$, $F_{2,54} = 6.143$, $r^2 = 0.016$, $P = 0.014$; Fig. 13). This indicates that the birds likely began molting at an earlier onset in addition to molting at a faster rate.

During normal molt, birds replace their flight feathers from the inner-most primary feather (P1) outward to the outer-most flight feather (P9 in starlings) (Pyle 1997). During molt assessment in my experiment, I observed that three birds in the 1.5 ppm MeHgCys -group (n = 3 out of 16) and one bird in the 0.75 ppm MeHgCys group (n = 1 out of 18) exhibited abnormal sequence of molt (Fig. 14). These birds dropped feathers out of sequence and were simultaneously growing new feathers. For example, in one case, P4 and P7 were being regrown but P5 and P6 had not yet been dropped. These four birds did not show the same molt aberration, but each had dropped feathers out of order, which does not normally happen in European Starlings. All birds in the control treatment (n = 19) showed normal sequential replacement of flight feathers. The rate of occurrence of this unusual molt pattern was not statistically significant (*Fisher's exact test*, $P = 0.158$). I did not note whether the disruption in molt sequence was asymmetrical or symmetrical in these birds (i.e. whether the same disruption occurred on just the right wing or the left wing also). Examining the symmetry of this molt disruption might be helpful in starting to understand the mechanisms of disruption.

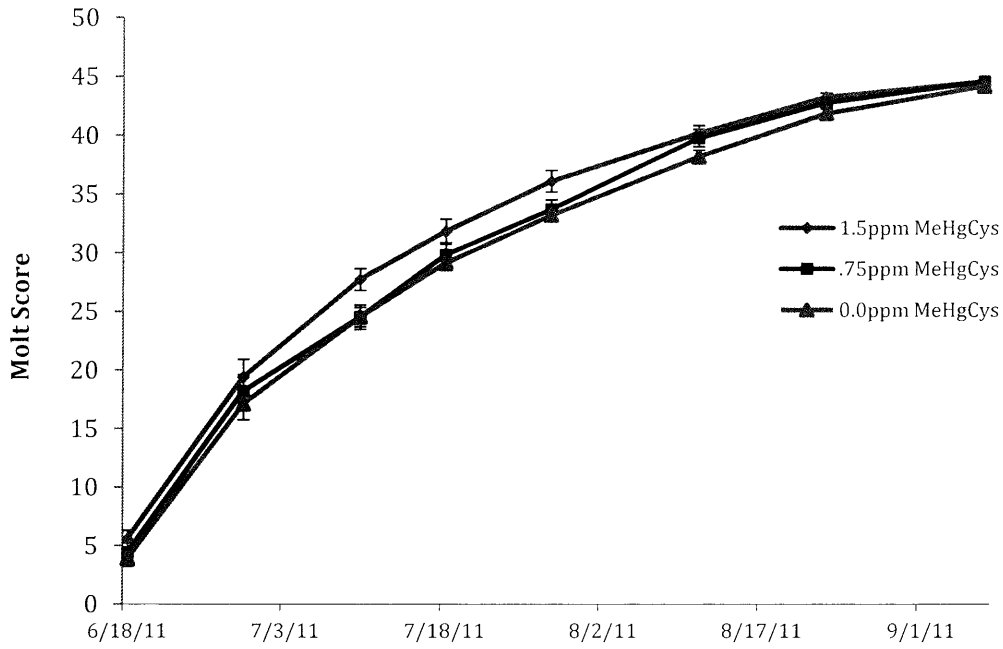


Figure 11: Average molt score across treatment groups from shortly after the onset of molt through to the end of molt ($F_{2,54} = 2.46$, $P = 0.09$).

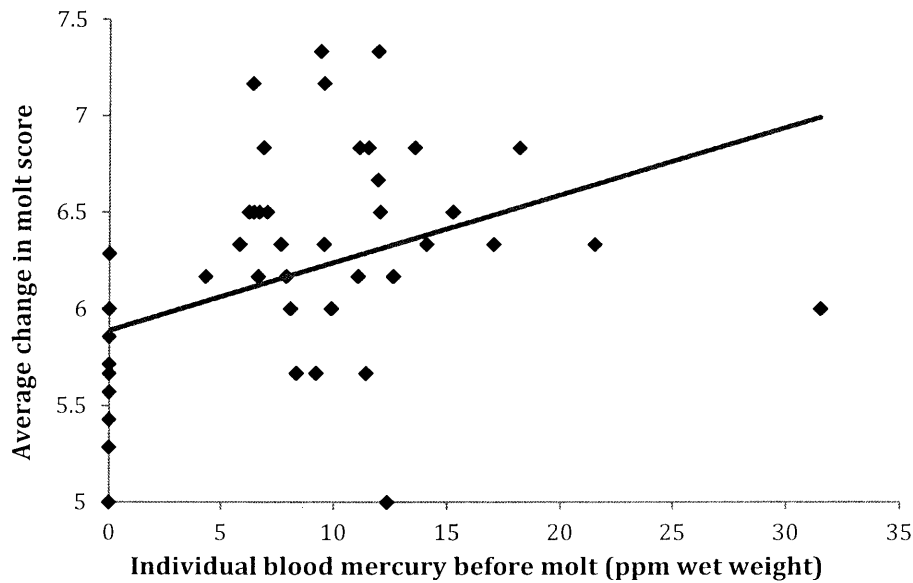


Figure 12: Relationship between blood mercury at the onset of molt (mid-May) and average change in molt score per scoring occasion (June through August 2011) ($y = 0.035x + 5.884$, $F_{2,55} = 10.133$, $r^2 = 0.16$, $P = 0.003$).

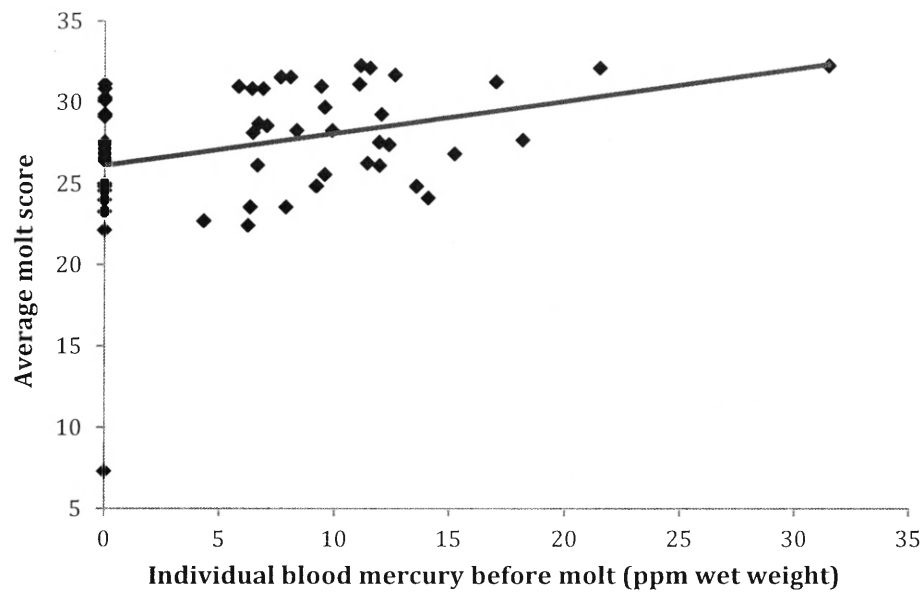
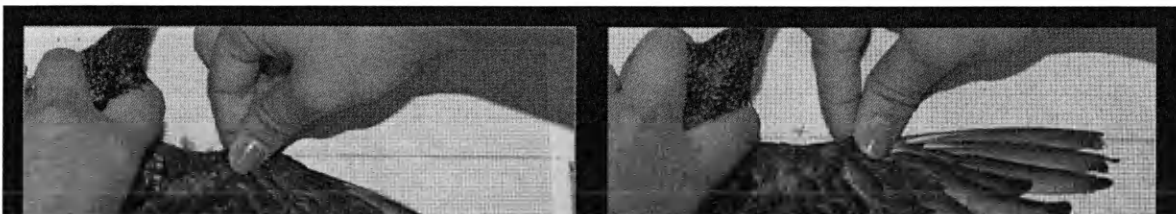


Figure 13: Relationship between blood mercury at the onset of molt (mid-May 2011) and average molt score (June through August 2011) ($y = 0.143x + 26.845$, $F_{2,54} = 6.143$, $r^2 = 0.016$, $P = 0.014$).



F

P9

P1

Figure 14: Photographs of right wings of a bird displaying a normal molt sequence from the control group (A) and a bird from the 1.5 ppm MeHgCys group showing abnormal molt sequence (B). Four birds on mercury but none of the control birds showed this disruption in flight feather molt sequence (*Fisher's exact test*, $P=0.158$).

DISCUSSION

Section I: Flight Performance

Chronic exposure to 1.5 ppm dietary mercury resulted in a general decrease in escape take-off flight performance. The most prominent differences occurred between session 1 (pre-dosing) and session 5 (40 weeks after dosing; 20 weeks after molt). Controls exhibited a 39.7% mean increase in energy gained during take-off between these two sessions, while birds dosed with 0.75 ppm MeHgCys demonstrated a relative mean decrease of 17.4% and birds dosed with 1.5 ppm MeHgCys exhibited a mean decrease of 31.3% (Fig. 5b). Furthermore, individual blood mercury was negatively related to change in energy expenditure (Fig. 6b), such that increasing blood mercury resulted in lowered energy expenditure during escape take-off. The overall pattern of results in the present study were consistent with the hypothesis that mercury causes decreased escape take-off flight performance.

Unexpectedly, there were differences in take-off performance among treatment groups before dosing began (See Fig. 4). Differences in cage environment are a possible explanation, but pseudoreplication is unlikely because the subjects were separated into their treatment groups (two cages per treatment) only 8 days prior to the session 1 flight test. Most likely there were actual differences among the groups resulting from normal variation combined with sampling error and low sample size. Although the initial difference in flight performance among the groups was not expected, the design of my experiment allowed for individuals to serve as their own controls, correcting for this problem. I analyzed within-individual changes among the groups, so the difference in

mean energy expenditure between treatments during session 1 did not affect the interpretation of my results.

Wild songbirds experiencing similar mercury exposure as the birds in the present study are likely to suffer direct fitness consequences as a result of decreased ability to escape predators (Lima 1993; Witter et al. 1994; Metcalfe and Ure 1995). There is also high potential for indirect fitness consequences resulting from deficiencies in foraging success, courtship, food provisioning to young, migratory journeys, or any number of vital behaviors in a songbird's lifetime that require efficient locomotion.

The specific cause behind the observed decrease in flight performance is not easily pinpointed because of the interconnectivity between physiological and neurological flight mechanisms (Pennycuik 1968, 1997; Biewener et al. 1992; Swaddle and Witter 1997; Dudley 2002; Tobalske et al. 2005) and the comprehensive effects of mercury on these systems (Eisler 2006; Cambier et al. 2009; Glaser et al. 2010; Seewagen 2010; Cai 2011). There may have been disruptions in several underlying flight mechanisms that led to the patterns I observed, and further research on mechanisms underlying this effect would be fruitful.

The consistent increase in energy expenditure demonstrated by control birds could signify that there were changes in some physiological flight mechanism that allowed for better performance over time. For example, captive starlings are able to adjust to captivity by regulating their mass in accordance to food availability (Wiersma et al. 2005). More specifically, captive starlings with unpredictable food availability expend more energy flying and maintain higher masses than birds exposed to a constant food source (Wiersma et al. 2005). Mass regulation and energy budget are directly related to

escape take-off flight performance (Swaddle and Witter 1997), thus the differential changes in flight performance between the groups may have resulted from a difference in perception of food availability, daily energy use, or mass regulation. Other differences in adaptive responses to captivity are also possible.

All the birds were exposed to successive anthropogenic stressors including frequent handling, which can reduce stress response in starlings (Dickens and Romero 2009) and subsequently decrease escape success. However, by 10 months in captivity, starlings typically reestablish a normal startle response. It is possible that the control birds were able to remount their typical stress response while the dosed birds were not, especially considering the known disruptive effects of mercury on corticosterone, a major mechanism for proper stress response in birds (Tan et al. 2009; Wada et al. 2009).

An alternative explanation for the increased performance exhibited only by controls is that control birds may have learned how to better complete the flight test over time while dosed birds did not. Mercury is known to cause cognitive deficiencies (Wolfe et al. 1998; Cai 2011), thus it is possible that there were differences in learning ability that led to increased performance by controls but not by dosed birds. My hypothesis that mercury-dosed birds were less capable of learning the flight test may also explain the changes in angle of take-off among the groups (Fig. 7a; Fig 7b). In an ecological setting, birds must make rapid decisions about the most effective angle and speed of take-off. Changes in angle over time could reflect changes in strategy based on previous encounters. Control birds took off at a significantly shallower angle in session 4 compared with session 1, but high-dosed birds did not appear to alter their strategy, taking off at only a slightly shallower angle (Fig. 7a). The predator stimulus in this simulation was terrestrial rather than aerial, so the birds may have originally perceived

the most efficient take-off response to be a steeper angle of trajectory, which has been shown to result in increased survival during attacks from terrestrial predators (Lima 1993; Witter et al. 1994). However, a typical wild predator would pursue the birds along the ground unlike the stationary predator stimulus used in the present study. Thus, it is possible that the control birds learned to alter their escape strategy by reducing their angle of take-off and reaching safety faster. The differences in change of angle from session 1 to session 5 were similar across all treatments (Fig. 7b). This may indicate that high-dosed birds required more exposure before learning to adjust to the test.

Differences between treatment groups in energy expenditure were not detectable during the molt period, although this is not surprising considering the costs that molt alone confers on take-off flight (Swaddle and Witter 1997). It is also important to note that while the birds were molting, mercury was being sequestered into their feathers (Condon and Cristol 2009), so the dosed birds were experiencing a much reduced mercury burden in living tissue during this period compared with the post-molt period (Fig. 3). Thus, any physiological effects of mercury, especially on a cellular level, may have been greatly reduced during molt.

Results from the maneuverability test revealed no differences in velocity or angle between the treatment groups from session A (pre-dosing) to session B (38 weeks after dosing, 16 weeks after molt). However, the maneuverability test was a novel approach to measuring turning flight performance, and the measurements taken were simpler than those taken in the take-off test. In the take-off test analysis, I combined velocity, height, and mass into a single metric of energy per unit mass (Swaddle et al. 1999), whereas I measured velocity and angle as separate variables to analyze turning flight performance.

In order to generate a measure of energy, I would have needed to use a second camera to record height. It is possible that tradeoffs between speed and angle rendered any effects of mercury on maneuverability flight undetectable.

In summary, birds in different treatment groups showed differential patterns of energy expenditure and strategy during escape-take off. In general, control birds improved in flight performance over time while low-dosed birds and high-dosed birds worsened. There are many mechanisms involved in flight, some of which are tightly interconnected. Thus, it is difficult to pinpoint which flight mechanisms may have been disrupted by mercury. But because these birds were not developmentally exposed, immediate effects of mercury on cellular activity may be likely. For example, mercury is related to increased oxidative stress (Henny et al. 2002), which is also magnified by engaging in an energetically expensive activity like flight. Reduced feather quality is also possible, which may have covaried with mercury treatment or occurred as a result of increased molt rate (discussed in Section III). It is likely that mercury caused alterations in a combination of many neurological, bioenergetic, physiological, and behavioral flight mechanisms (Hoffman and Heinz 1998; Bouten et al. 2000; Eisler 2006; Evers 2008; Franceschini et al. 2009; Frederick and Jayasena 2011) that together reduced flight performance.

Based on my results, I would recommend further testing to begin to understand whether cognitive function and/or physiological changes are influencing flight performance. For example, applying differential flight tests with unique predator stimuli on each testing occasion could reduce the ability of birds to learn to perform the test better but still show changes in energy expenditure during simulated escape. Applying a

flight test that requires learning in parallel to this would allow for the differentiation between effects of mercury on learning and effects of mercury on some other physiological parameter during escape flight.

I would also recommend tests that better elucidate what cellular mechanisms may be causing the observed changes. For example, it is believed that corticosterone increases energy levels for a prolonged period after a stressor is presented, which allows an animal to recover from a stimulus quickly and respond to a subsequent stressor (Sapolsky et al. 2000). Therefore, exposing birds to successive predator stimulus simulations may elucidate differences in corticosterone mediated stimulus response. Differences in the ability to recover from oxidative stress would also be apparent.

In summary, mercury dosed birds suffered a consistent decrease in escape take-off flight performance. Considering the number of predator-prey interactions that occur during the lifetime of a wild bird along with the myriad of important behaviors that require efficient flight ability, it is possible that this decrease would have significant fitness effects. It is important to recognize that the magnitude of the observed reduction in flight performance would likely be exaggerated in wild populations because wild birds undergo much more day to day stress than captive birds.

Section II: Molt

Molt rate and average molt score increased with blood mercury across treatments (Figures 12, 13). These results indicate that mercury caused birds to molt faster. Further investigation is needed to elucidate whether mercury dosed birds also experienced an earlier date of onset. An increased rate of molt is of general ecological significance because it can result in poor-quality feathers, which negatively affect flight and thermoregulatory ability (Dawson et al. 2000). Changes in the timing of molt are also important because molt phenology plays a central role in the energetic budgets of birds (Renfrew 2011). Birds undergo molt when they are least likely to engage in other energetically expensive activities such as breeding, parenting, or migration. If the temporal relationship between any of these events were altered or if molt were to overlap with them, there may be deleterious fitness consequences (Echeverry-Galvis 2012).

Since reduced feather quality can cause decreased flight performance, the observed increased rate of molt among mercury-exposed birds could play a role in explaining their associated decrease in take-off flight performance. Specifically, the dosed birds may have grown poor quality feathers as a result of accelerated molt, which consequently caused a reduction in flight performance. However, it is important to note that during session 1 the starlings, which were all juveniles, had not yet undergone their first pre-basic molt. Therefore their flight feathers had been experiencing wear for nearly 8 months. During sessions 4 and 5 newly grown flight feathers had only been experiencing wear for about half that amount of time, but feathers of poor initial quality are more vulnerable to degradation (Vagasi 2011).

During the molt assessment, I observed out of sequence feather loss in four starlings dosed with mercury (Fig. 14). During normal molt in starlings, primary feather loss is staggered so that birds do not have large gaps in their flight feathers at any one

time; this helps to ameliorate the reduction in flight performance caused by molt (Dawson 2003). Out of sequence feather loss and the simultaneously re-growing of feathers creates abnormally large gaps and could affect flight and other functioning of the wing. The number of birds exhibiting this abnormality in my study was not statistically significant, yet to my knowledge this kind of alteration has never been recorded despite abundant research on molt in starlings (J. P. Swaddle, unpublished data). Unfortunately, I did not examine the symmetry of this molt disruption, which would have clarified whether this observation indicated a systematic problem with the control of molt or local injuries or aberrations that affected just a few feathers on one wing.

Changes in hormonal fluxes are thought to drive timing and rate of molt, and have also been associated with mercury in a variety of studies (Burton & Meikle 1980; Hontela et al. 1992; Friedman et al. 1998; Leblond & Hontela 1999). Thus, disturbances in steroidogenesis may have resulted in differential molt patterns between the dosed and control birds. One specific hypothesis is that mercury depressed testosterone (Frederick and Jayasena 2011), which is closely linked with the onset of molt in starlings (Nolan et al. 1992). But it is important to note that the birds used in my study were non-breeding, non-migratory captive birds, so they were likely not experiencing the same fluxes in hormonal levels that wild birds experience. Molt occurred earlier than it does in the wild for all of the captive birds, presumably because the absence of reproductive behavior lowered circulating testosterone. Tracking the progress of molt in wild birds environmentally exposed to mercury would be necessary to determine whether the differences I observed in captivity are repeatable in wild populations experiencing normal hormonal fluxes.

Overall, results from the molt assessment demonstrate that mercury affected molt pattern. Birds with higher blood mercury molted at a faster rate and, on occasion, molted in a pattern that is highly unusual for starlings. These results are ecologically relevant because increased molt rate leads to reduced feather quality and decreased survival (Dawson 2002; Hinsley et al 2003).

Section III: Environmental Relevance of Induced Mercury Levels

A limited number of studies have measured blood mercury in passerine birds, especially those that forage primarily in terrestrial ecosystems. Based on the data available, it is reasonable to assume that the blood mercury levels induced in this study (4.2 ppm – 11.3 ppm) are most likely reflective of birds exposed to high mercury contamination at an industrial point source. For example, Cristol et al. (2008) recorded blood mercury levels in Carolina Wrens (*Thyrothorus ludovicianus*) living in a point-source location that averaged 8.76 ± 6.46 (SD) ppm. However, most other studies have reported much lower levels of blood mercury in passerines (Rimmer 2005; Eisler 2006) even in areas contaminated with mercury (Brasso et al. 2008; Condon and Cristol 2009; Jackson et al. 2011). More research is needed to better understand how environmental mercury relates to accumulation in terrestrial birds, especially considering differences in foraging behavior, dietary intake, metabolism, and body size among species.

It is also useful to consider mercury levels of terrestrial invertebrates living in contaminated areas since they are a common food source for many songbirds. Cristol et al. (2008) reported that spiders consumed by passerines in a contaminated area had an

average total mercury concentration of 1.24 ± 1.47 ppm dry weight, which is similar to the lower dose used in the present study (equivalent to 1.83 ppm dry weight). However, spiders contained elemental mercury in addition to methylmercury, so actual methylmercury consumed by predators would be lower. Much lower mercury levels were reported in invertebrates sampled from an array of ecosystems across North America (0.21 - 0.28 ppm dry weight in areas with mercury present in the soil; Hargreaves et al. 2011).

In summary, the blood levels induced in the present study are higher than levels reported for songbirds in most cases, but can occur in some areas of high contamination. There is also some indication that passerine prey items in areas contaminated by point-source mercury have similar proportions of mercury to the food used to dose the birds in my study, although a portion of the mercury is elemental. Based on these limited associations, the implications of this study are likely most applicable to songbirds living in point-source contaminated areas with relatively high environmental mercury contamination. Terrestrially foraging passerines exposed to lower-levels of environmental mercury through atmospheric deposition are not likely experiencing the effects demonstrated here.

Section IV: Conclusion

There have been many studies that assess the effects of mercury on the health of wildlife, however little is known about the effects of mercury on passerine birds, a terrestrial taxa common in areas where mercury is present throughout the food chain. Free-living passerines with high levels of mercury in blood and feathers are known to suffer impaired reproductive success (Brasso and Cristol 2008; Jackson et al. 2011), so it is likely that upstream responses to mercury are affecting a multitude of important health parameters. In this study I found that starlings chronically exposed to dietary mercury had abnormal molt patterns and decreased the energy expended during escape take-off, both of which could affect fitness. This research is aimed at beginning to fill in the knowledge gap concerning risk thresholds for terrestrial songbirds, and indicates that 1.5 ppm dietary MeHg may pose threats to songbirds by altering flight performance and molt.

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